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# Effect of Pyridines on Phenotypic Properties of *Bordetella pertussis*

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Several conditions of growth of *Bordetella pertussis* cause a reversible phenotypic alteration in properties termed modulation. Growth in medium containing nicotinic acid induces normal (X-mode) cells to change to modulated (C-mode) cells. We examined several pyridines and compounds resembling pyridines for their ability to affect modulation, using envelope protein patterns and serological reactivity as indicators of modulation. We found that 6-chloronicotinic acid and quinaldic acid were more effective modulating stimuli than was nicotinic acid on a molar basis. Both 2-chloronicotinamide and isoniazid interfered with nicotinic acid-induced modulation, and can be called antimodulators. Picolinic acid inhibited growth.

*Bordetella pertussis*, the causative agent of whooping cough, is a gram-negative bacterium which characteristically demonstrates several biological activities. These include (i) a dermonecrotic toxin (2), (ii) an extracellular adenylate cyclase (10), (iii) a filamentous hemagglutinin (1), and (iv) a complex of activities variously termed lymphocytosis-promoting factor, histamine-sensitizing factor, islet-activating protein, soluble hemagglutinin, or pertussigen (17, 18, 26). The latter two activities appear to be associated with the cell envelope and may be protective components in pertussis vaccine (11, 22, 25).

Upon repeated subculture in vitro, *B. pertussis* may undergo a spontaneous loss of some or all of these activities as well as of immunogenicity (9). This irreversible process is known as degradation and is accompanied by a specific change in envelope proteins (19, 25). Other changes associated with degradation include loss of cytochrome *d* (4) and an alteration in serological reactivity (15). Degradation may be mimicked by a reversible phenotypic change known as modulation. Lacey (13) introduced the term X-mode (for xanthic mode) and C-mode (for cyanic mode) to describe normal and modulated bacteria, respectively. Modulation of *B. pertussis* is induced by growth in medium containing high levels of magnesium or by growth at low temperature (13). Exogenous NA, supplied to meet the requirement of *B. pertussis* for NA or Nam (12, 21), has also been shown to induce changes resembling modulation (20, 24). Modulation, unlike degradation, is readily reversible by growth under normal cultural conditions. The characteristic changes in envelope proteins which accompany modulation appear identical to those seen in degradation (4, 6, 19, 24, 25). Understanding modulation may have practical benefits for development of methods to stabilize the expression of protective components in vaccines or to enhance expression of these components. On a broader level, understanding modulation may provide general insight into the regulation of synthesis of envelope proteins of gram-negative bacteria.

We examined the ability of several pyridines and related compounds to induce modulation of *B. pertussis* and to interfere with NA-induced modulation, using antibody reactivity and envelope protein patterns to evaluate the effects of the compounds.

## MATERIALS AND METHODS

**Abbreviations.** The following abbreviations are used in this paper: NA, nicotinic acid; Nam, nicotinamide; TIE, Triton X-100–insoluble envelope.

**Bacterial strains.** A clone from a clinical isolate of *B. pertussis* UT25 obtained by two sequential single-colony isolations at laboratory passage 5 was used (8).

**Reagents.** 3-Acetyl-pyridine, isoniazid, isonicotinic acid, Nam, NA, picolinic acid, quinaldic acid, and quinolinic acid were purchased from Sigma Chemical Co., St. Louis, Mo.; 2-chloronicotinamide, 6-chloronicotinamide, 2-chloronicotinic acid, 6-chloronicotinic acid, methyl nicotinate, and pyridine-3-sulfonic acid were obtained from Aldrich Chemical Co., Milwaukee, Wis.

**Culture conditions.** Basal medium was modified from the liquid minimal medium of Stainer and Scholte (23) and contained the following (in grams per liter):

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NaCl, 2.5; $KH_2PO_4$, 0.5; KCl, 0.2; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.01; Tris, 1.52; sodium glutamate, 10.72; L-proline, 0.24; L-cystine, 0.04; glutathione, 0.01; ascorbic acid, 0.02; Nam, 0.004; pH to 7.6 with HCl. Vitamins, glutathione, and $FeSO_4 \cdot 7H_2O$ were filter sterilized and added to autoclaved medium. Cells were grown in acid-cleaned 500-ml Erlenmeyer flasks containing 200 ml of medium for 22 to 26 h in a shaking incubator (200 rpm) at 35°C. Pyridine compounds were dissolved in water, filter sterilized, and added to yield a final concentration of 500 μg/ml unless otherwise stated. Modulation by $Mg^{2+}$ was accomplished by subculturing cells twice in basal medium supplemented with 20 mM $MgCl_2$ (6).

**Envelope preparation.** A modification of the Triton X-100 extraction procedure of Diedrich et al. (3) was used. Cells were harvested by centrifugation at 3,300 × *g* for 20 min, suspended in 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4), and stored at −70°C until use. Cells were broken by sonication, using a Heat Systems model W-375 sonicator with microtip attachment set on the 50% pulse mode and power setting 2. Sonication was for 15 min, with continuous cooling in an ethanol-ice bath. Unbroken cells were removed by centrifugation at 3,000 × *g* for 10 min. The supernatant was centrifuged at 107,000 × *g* for 1 h, and the pellet from this centrifugation was homogeneously suspended in a solution of 2% Triton X-100 and 7.5 mM $MgCl_2$ in 0.05 M HEPES buffer, pH 7.4. After standing for 30 min at room temperature, the preparation was centrifuged for 1 h at 107,000 × *g*, and the pellet was then suspended in distilled water. This Triton X-100–insoluble material is referred to as the TIE fraction and appears to correspond to the outer envelope (6).

TIE preparations (at 1.5 mg of protein per ml) were solubilized in a mixture of 12.5% glycerol, 1.25% sodium dodecyl sulfate, 1.25% 2-mercaptoethanol, 0.5 M urea, and 0.005% bromophenol blue in 0.25 M Tris-hydrochloride buffer (pH 6.8) by boiling for 5 min. Urea was necessary to successfully disassociate C-mode envelope polypeptides within the 5-min boiling period.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** A 7.5 to 20% gradient gel was employed, using a Laemmli (14) discontinuous buffer system modified to contain 0.5 M urea (Bio-Rad Laboratories, Richmond, Calif.) in the stacking and resolving gels. Gel dimensions were 0.75 mm by 12 cm by 14 cm. A 15- to 20-μg amount of protein (determined by the method of Lowry et al., using bovine serum albumin as a standard [16]) was applied per lane. Electrophoresis was performed at 0.6 W on the constant power mode for 18 h. Molecular weight markers were from Bio-Rad Laboratories. Staining and destaining in Coomassie blue R-250 were done by the method of Fairbanks (7).

**Preparation of antisera and agglutination tests.** Vaccines were prepared by suspending harvested X-mode or NA-induced C-mode cells in nonpyrogenic saline (Travenol Laboratories, Deerfield, Ill.) containing 0.001% thimerosal at a protein concentration of 400 μg/ml for X-mode cells and to 900 μg/ml for C-mode cells. Antisera were prepared in rabbits by six weekly injections. An initial subcutaneous injection of 0.4 ml of vaccine emulsified in 0.4 ml of Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich.) and given in equal amounts at four sites was followed 7 days later by intravenous injection of 0.2 ml of vaccine. This scheme was repeated twice, and the rabbits were bled. Preimmunization serum was negative for agglutinating antibody to X-mode or C-mode *B. pertussis* cells.

Antiserum agglutination titers were determined by a modification of a previously described method (18). Harvested cells were washed once with 0.9% NaCl and suspended in saline at a reading of 150 Klett units (Klett-Summerson colorimeter; green filter). Samples (0.1 ml) of the cell suspension were added to 0.1-ml volumes of serially diluted antiserum. After mixing, tubes were incubated at 37°C for 1 h and held at 4°C overnight before reading. A difference in agglutination titer of more than fourfold (two tubes) was considered significant.

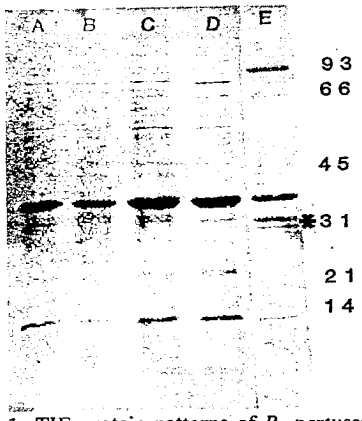


FIG. 1. TIE protein patterns of *B. pertussis* after electrophoresis in polyacrylamide gradient gels containing sodium dodecyl sulfate and urea. (A) Cells grown twice in basal medium containing 20 mM added $MgCl_2$. (B) Cells grown in basal medium containing 500-μg/ml NA. (C) Cells grown in basal medium containing 500-μg/ml 6-chloronicotinic acid. (D) Cells grown in basal medium containing 500-μg/ml quinaldic acid. (E) Cells grown in basal medium. The asterisk indicates the doublet at about 30,000 to 32,000 molecular weight, which is characteristic of X-mode cells. The positions of molecular weight markers are shown. Markers included phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

## RESULTS

**Modulation by NA.** The appearance of TIE protein preparations from X-mode cells, cells modulated by $Mg^{2+}$, and cells modulated by NA is shown in Fig. 1, lanes E, A, and B, respectively. Preparations from cells grown twice in high $Mg^{2+}$ or once in NA showed loss of two proteins in the 30,000 to 32,000 range—a pattern characteristic of both modulation and degradation.

TABLE 1. Agglutination titers of *B. pertussis* in anti-X-mode and anti-C-mode sera when grown in the presence of pyridines[a]

| Test pyridine present in basal medium | Addition of NA[b] | Classification of test pyridine | Agglutination titer with following serum: | |
|---|---|---|---|---|
| | | | Anti-X-mode | Anti-C-mode |
| None | − | | 512 | 16 |
| | + | | 16 | 128 |
| 6-Chloronicotinic acid | − | Modulator | 32 | 128 |
| | + | | 32 | 64 |
| Quinaldic acid (quinoline-2-carboxylic acid) | − | Modulator | 16 | 128 |
| | + | | 16 | 128 |
| 2-Chloronicotinamide | − | Antimodulator | 512 | 16 |
| | + | | 512 | 64 |
| Isoniazid (isonicotinic acid hydrazide) | − | Antimodulator | 256 | 16 |
| | + | | 256 | 32 |
| Picolinic acid (2-pyridinecarboxylic acid) | − | Growth | 256 | 32 |
| | + | inhibitor[c] | 64 | 64 |
| 3-Acetylpyridine | − | No effect | 512 | 16 |
| | + | | 32 | 64 |
| 6-Chloronicotinamide | − | No effect | 128 | 16 |
| | + | | 64 | 64 |
| 2-Chloronicotinic acid | − | No effect | 1,024 | 32 |
| | + | | 32 | 32 |
| Isonicotinic acid (4-pyridinecarboxylic acid) | − | No effect | 256 | 16 |
| | + | | 32 | 128 |
| Methyl nicotinate | − | No effect | 512 | 16 |
| | + | | 64 | 128 |
| Nam (3-pyridinecarboxylic acid amide) | − | No effect | 512 | 16 |
| | + | | 64 | 64 |
| Quinolinic acid (pyridine-2,3-dicarboxylic acid) | − | No effect | 512 | 16 |
| | + | | 32 | 128 |

[a] *B. pertussis* UT25 was grown in basal medium containing test pyridines at a final concentration of 500 μg/ml for 22 to 26 h at 35°C. *B. pertussis* cells were suspended in saline at a standard density, and tube agglutination tests were performed, using unabsorbed antibody made against X-mode and NA-induced C-mode cells.

[b] NA is 3-pyridinecarboxylic acid and was added at 500 μg/ml (4.06 mM).

[c] Picolinic acid was tested at a 50-μg/ml final concentration due to its inhibition of growth at higher concentrations.

Cells grown in $Mg^{2+}$ or NA reacted poorly in antisera prepared against normally grown (X-mode) cells, whereas cells grown normally reacted poorly in antisera prepared against NA-grown cells (Table 1). NA-grown cells also showed very low levels of dermonecrotic toxin activity, histamine-sensitizing factor activity, and lymphocytosis-promoting factor activity (data not shown). From these results, we concluded that our strain underwent typical modulation when grown in high-$Mg^{2+}$ or high-NA medium.

**Ability of other pyridines to induce modulation.** Cells grown in basal medium containing added pyridines were harvested and examined for TIE patterns and serological reactivity. Since *B. pertussis* requires NA or Nam for growth (12, 21), 4.0 μg of Nam per ml was always present in growth medium. Test pyridines included both isomers of NA (isonicotinic acid and picolinic acid), a dicarboxypyridine (quinolinic acid), a carboxyquinoline (quinaldic acid), and seven variously substituted pyridines (Table 1). Surprisingly, of the compounds tested, only pico-

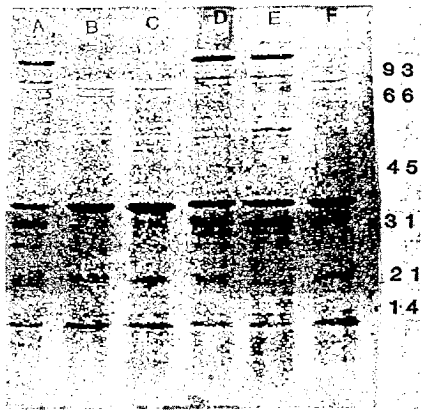


FIG. 2. TIE protein patterns of *B. pertussis* grown at various concentrations of modulation inducing pyridines. (A) NA, 1 mM. (B) 6-Chloronicotinic acid, 1 mM. (C) Quinaldic acid, 1 mM. (D) NA, 0.1 mM. (E) 6-Chloronicotinic acid, 0.1 mM. (F) Quinaldic acid, 0.1 mM. Cells were grown in basal medium supplemented with pyridines as shown. The positions of marker proteins (as in Fig. 1) are indicated. Note that lanes B, C, and E lack both the 30,000- to 32,000-molecular-weight doublet and a high-molecular-weight polypeptide (ca. 110,000). A polypeptide doublet occurs at about 25,000 in lanes A, D, and F, whereas only a single band is seen at that region in lanes B, C, and E. Differences in polypeptides in the 60,000 to 90,000 range are also seen.

linic acid markedly inhibited growth. A 50-μg/ml amount of picolinic acid was chosen as a test level for this compound.

Two compounds, 6-chloronicotinic acid and quinaldic acid, induced the same changes in envelope proteins and serological reactivity as were caused by NA (Fig. 1, lanes C and D; Table 1). No other test pyridine induced any change in TIE or serological reactivity. The ability of 6-chloronicotinic acid and quinaldic acid to cause modulation was probably not due to breakdown in vivo to NA since neither compound was capable of supporting growth of *B. pertussis* when Nam was omitted. When compared with NA on a molar basis, both compounds were more effective modulating agents (Fig. 2 and Table 2). The lowest NA concentration yielding C-mode TIE patterns was 4 mM. 6-Chloronicotinic acid at 1 mM or quinaldic acid at 0.1 mM induced C-mode TIE patterns. By serological criteria, 4 mM NA was required for modulation, but 1 mM 6-chloronicotinic acid or 0.1 mM quinaldic acid was sufficient.

**Ability of pyridines to prevent NA-induced modulation.** Cells grown in basal medium plus NA were also supplemented with other pyridines. Both isoniazid and 2-chloronicotinamide interfered with NA induced modulation. Cells grown with NA and either 2-chloronicotinamide or isoniazid showed high serological reactivity with anti-X-mode sera and normal reactivity with anti-C-mode sera (Table 1). Figure 3 shows the TIE patterns of cells grown in NA plus each of these modulation inhibitors. Cells grown with isoniazid plus NA retained X-mode proteins but showed an increase in the relative amount of a 36,000-molecular-weight protein, a pattern characteristic of C-mode cells. Cells grown in 2-chloronicotinamide plus NA showed a typical C-mode TIE pattern despite retention of X-mode

TABLE 2. *Agglutination titers of B. pertussis in anti-X-mode and anti-C-mode sera when grown with various amounts of pyridine modulators*[a]

| Pyridine added | Final concn (mM) | Agglutination titer with following serum: | |
|---|---|---|---|
| | | Anti-X-mode | Anti-C-mode |
| Nicotinic acid (3-pyridinecarboxylic acid) | 0.001 | 512 | 16 |
| | 0.01 | 512 | 16 |
| | 0.10 | 512 | 32 |
| | 1.00 | 256 | 64 |
| | 4.00 | 16 | 128 |
| 6-Chloronicotinic acid | 0.001 | 512 | 16 |
| | 0.01 | 256 | 16 |
| | 0.10 | 256 | 64 |
| | 1.00 | 64 | 128 |
| Quinaldic acid (quinoline-2-carboxylic acid) | 0.001 | 256 | 16 |
| | 0.01 | 256 | 16 |
| | 0.10 | 16 | 128 |
| | 1.00 | 16 | 128 |

[a] *B. pertussis* UT25 was grown in basal medium containing pyridines at the concentrations shown for 20 to 22 h at 35°C. *B. pertussis* cells were suspended in saline at a standard density, and tube agglutination tests were performed, using unabsorbed antibody made against X-mode and NA-induced C-mode cells.

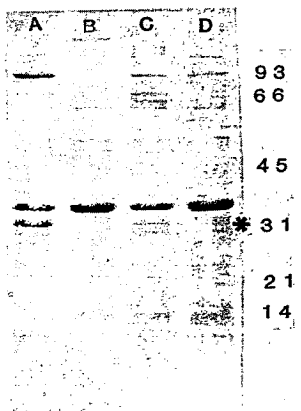


FIG. 3. TIE protein patterns of *B. pertussis* grown in the presence of antimodulators. (A) 2-Chloronicotinamide; (B) NA plus 2-chloronicotinamide; (C) isoniazid; (D) NA plus isoniazid. Cells were grown in basal medium supplemented with pyridines at 500-μg/ml final concentration. Note that lanes A and B show X-mode and C-mode patterns, respectively, whereas lanes C and D both give patterns like X-mode cells, but with decreased quantities of the 30,000- to 32,000-molecular-weight doublet. The 36,000-molecular-weight major protein in D is somewhat enhanced. Marker proteins are as in Fig. 1.

serological reactivity. Typical C-mode TIE patterns were seen for cells tested after growth in NA plus each of the other pyridines (data not shown).

## DISCUSSION

These results indicate that several pyridine compounds and a quinoline compound are able to induce modulation of *B. pertussis*. Both of the newly reported modulators share some structural features of NA, having a pyridine ring which is substituted with a carboxyl group in position 2 or 3. The 2-substituted isomer of NA (picolinic acid) inhibits growth, and could not be tested adequately for modulating activity, but the 4-substituted isomer of NA did not induce modulation at the level tested. Modification of the carboxyl group by methylation (methylnicotinate) or acetylation (3-acetylpyridine) abolished the modulating activity of NA. However, 2,3-dicarboxypyridine (quinolinic acid) did not induce modulation.

On a molar basis, both 6-chloronicotinic acid and quinaldic acid were more effective modulators than NA, suggesting that substitution at position 5 or 6 may enhance modulation activity. Alternatively, such substitution may prevent the breakdown of the pyridine, making it apparently more effective. The rank order of the modulation efficiency of the compounds we have tested was as follows: quinaldic acid (most effective) > 6-chloronicotinic acid > NA > $Mg^{2+}$.

The ability of other pyridines to interfere with NA-induced modulation was also demonstrated. Isoniazid and 2-chloronicotinamide, when present with NA in the growth medium, were able to interfere with NA-induced modulation. It is notable that both of these compounds show structural similarities to Nam. However, neither Nam nor 6-chloronicotinamide was able to prevent modulation at the levels tested. This observation suggests that anti-modulators resemble Nam, but that Nam itself is not the most effective antimodulator. It is possible that antimodulators and even Nam might be more effective if tested at higher concentrations. Since the basal medium contained 4 μg of Nam per ml and the test involved adding an additional 500 μg/ml, Nam was examined at more than 100 times the NA concentration.

One may wonder also whether NAD is an antimodulator. Addition of NAD to the basal medium led to the growth of cells which expressed properties of both X-mode and C-mode (Schneider and Parker, manuscript in preparation). Thus, exogenous NAD was neither a modulator nor an antimodulator. Rather, it affected expression of cell properties in a unique way.

The antimodulators affected serological reactivity, although TIE patterns were changed little, if at all. This observation suggests that agglutination of cells by antisera measures surface properties or antigens different from the proteins detected by TIE electrophoresis. Earlier workers (5) have noted that protein antigens do not necessarily correspond to agglutinins.

These results also show that chelation of metals by pyridine compounds is not responsible for modulation since the chelation properties of the pyridines were unrelated to their ability to induce modulation.

The occurrence of antimodulators may be of value in vaccine production since it is likely that the same processes which regulate *B. pertussis* modulation regulate the synthesis of protective antigens and toxic components.

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